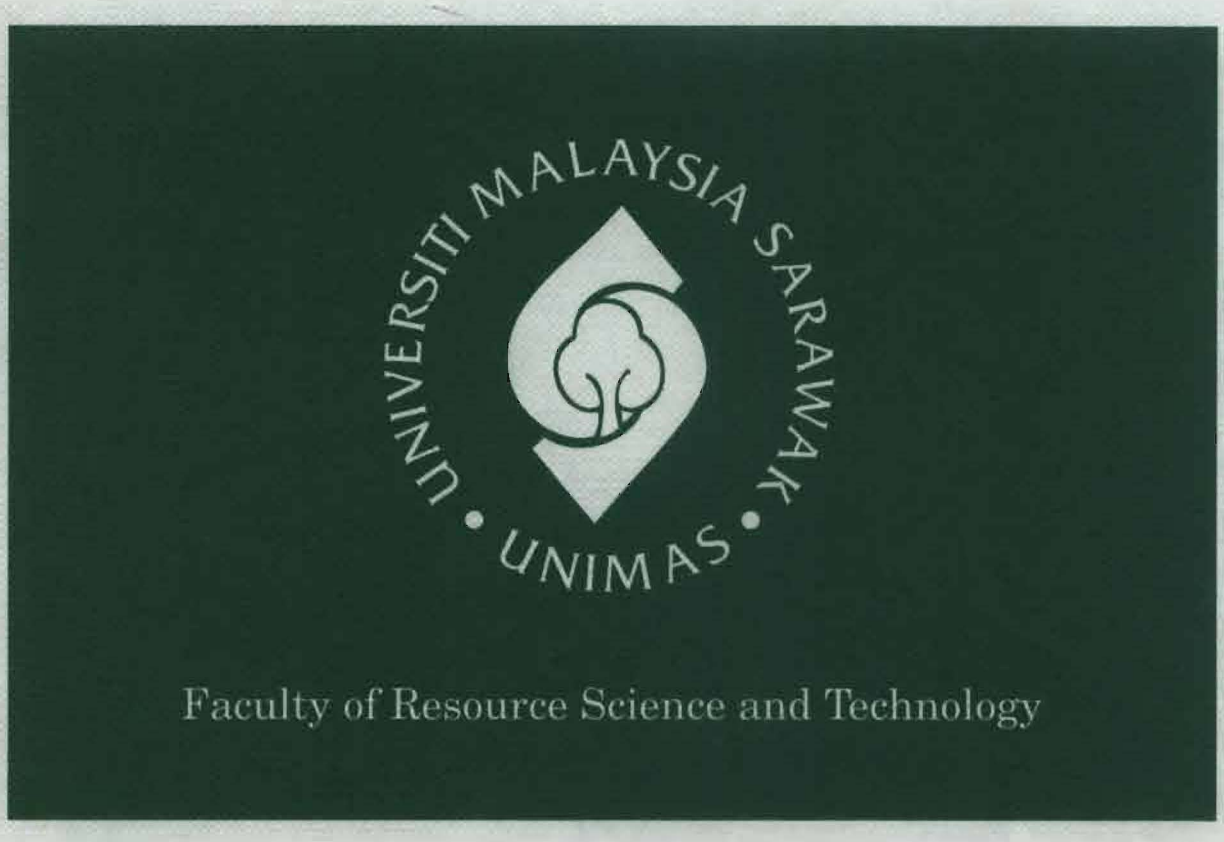


# SYNTHESIS OF FIRST STRAND cDNA FROM *Metroxylon sago* FLOWER TISSUE

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# Synthesis of first strand cDNA from *Metroxylon sago* flower tissue



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A report submitted in partial fulfilment of the requirements for the
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**Resource Biotechnology Programme**
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# Synthesis of First Strand cDNA from *Metroxylon sagu* Flower Tissue

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## ABSTRACT

Sago is well known for its starch producing trunk which becomes a basic food source for the locals especially in Sabah and Sarawak. However, little is known about their molecular characteristic. So, this study will emphasize on the construction of cDNA library from flower tissue. With the generation of a cDNA library it is hoped that it could later assist in a comparative study since the gene activities are different in every cell types. The generation of a cDNA library would require abundant amount of mRNA molecules. This can be achieved by extracting total RNA from the inflorescence. The total RNA was successfully extracted and the first strand cDNA was synthesized with the presence of AMV reverse transcriptase enzyme. Alas, the cDNA library cannot be finished in time due to time restraint and in short supply of reagents.

Key words: Inflorescence, cDNA library, total RNA, AMV reverse transcriptase.

## *ABSTRAK*

*Sagu sudah sememangnya terkenal dengan penghasilan kanji di dalam batangnya. Kanji yang terhasil telah menjadi makanan ruji bagi penduduk tempatan terutama sekali di kawasan Sabah dan Sarawak. Akan tetapi, pengetahuan tentang karektor molekularnya sangatlah terhad. Oleh itu, kajian yang telah dijalankan akan memfokus ke arah pembikinan perpustakaan cDNA daripada tisu bunga atau dikenali juga sebagai mayang. Dengan ini, ia diharapkan dapat membantu di dalam kajian perbandingan yang akan datang, memandangkan aktiviti gen bagi setiap jenis tisu adalah berbeza. Pembikinan perpustakaan cDNA memerlukan kuantiti molekul mRNA yang banyak. Ini hanya akan dapat dicapai dengan pengekstrakan total RNA daripada tisu mayang. Akhirnya, total RNA telah berjaya diekstrak dan pembentukan rantaian pertama cDNA telah berjaya dilakukan dengan kehadiran enzim AMV reverse transcriptase. Namun begitu, pembikinan perpustakaan cDNA tidak sempat disiapkan kerana kesuntukan masa dan kekurangan reagen.*

*Kata kunci: Mayang, perpustakaan cDNA, total RNA, AMV reverse transcriptase.*

# 1. INTRODUCTION

*Metroxylon sagu* or sago palm is said to be originated from New Guinea and the Moluccas. However, it has recently been dispersed across South-East Asia and the nearby Pacific islands. Sago palm grows well in Malaysia especially in Sabah and Sarawak and some parts in Peninsula which becomes a staple food for the locals as it produces starch in the trunk (Anon., 2004). The uniqueness about sago palm is, it only flowers once after 12 to 15 years of age and then it dies (Saidin, 1993), thus makes it interesting to study its molecular properties. Up till now, little is done about the flowers of sago for molecular studies especially in constructing cDNA libraries.

To extent, complementary DNA or cDNA is a molecule that results from a special enzyme activity called reverse transcriptase, which complements the base sequence in messenger RNA (mRNA). This particular enzyme is originally isolated from retroviruses (Alcamo, 1996). However, mRNA strand itself cannot be ligated directly into a cloning vector such as *E. coli* or lambda phage. It can only be ligated into the vector after a DNA polynucleotide is synthesized complement to the existing RNA molecule (Brown, 1992). Once the first-strand of cDNA is synthesized, the process is continued by DNA polymerase to produce a double-stranded cDNA molecule. Usually, lambda phages such as λZAP phage are more favourable as a vector because phage heads will automatically selects and package a chromosome about 50kb in length (Griffiths *et al.*, 1999).

Subsequently, a collection of cells that have a specific set of genes incorporated into their plasmid for later use is called a cDNA library (Alcamo, 1996). The cDNA libraries play an important role in keeping identified gene of interest when it is expressed in certain parts of the tissue or cell type (Brown, 1992). The genes are then can be obtained prior to use and it is relatively an easy task to screen the cDNA library for the clone that carries

the desired gene (Brown, 1992). Although it sounds easy to generate cDNA clones theoretically, cDNA clones are technically extremely difficult to generate that really represents the full length of mRNA (Glover, 1984). Besides, mRNA molecules are easily degradable, inherently instable and very sensitive (Walker & Rapley, 1997).

Ultimately, the primary objective of this study is to construct a cDNA library of *Metroxylon sagu* taken from flower tissue. Since the gene activities are different in every cell types of the same organism (Russell, 1992), it is significant to generate cDNA library in order to assist in a comparative study. Moreover, with the construction of *M. sagu* cDNA library, it is hoped that other researchers will proceed to further explore the functional genomics and associate proteins. Up till now, there is not much research based on construction of cDNA library especially from flower tissue.

## 2. LITERATURE REVIEW

### *2.1. Sago palm*

Sago palm or scientifically known as *Metroxylon sagu* grows well in peat soils. Sago, a medium to tall tree, grows from seedlings into a rosette stage of leaves and forms trunks after 4 to 6 years of growth (Jong, 1995). The sago life cycle reaches its ultimate stage with the development of a huge inflorescence which begins after 4 to 14 years of trunk formation (Jong, 1995). The trunk of sago palm is about 30 to 60cm in diameter (Anon., 2004) and it contains starch that act as a food reservoir for flowering and fruiting (Flach, 1997). The trunk of the tree can reach up to 7 to 25m high (Anon., 2004). Usually, sago trees for commercial used are cut down before the flowering season and the stored starch are extracted by shaking it under running water. Each trunk can yield about 250-650 lbs of starch which is in forms of sago pearls (Anon., 2004).

Flowering only occurs once and in the early stage of development, flower buds are arranged in pairs in a bracteole of one staminate (male) and the other is a hermaphrodite (perfect) flower. The inflorescences are in terminal panicles, up to 5 to 7m high (Anon., 2004) as shown in Figure 1 below. However, at some stage in the development, one of the flower buds (either the staminate or the hermaphrodite flower) will be aborted leaving only one type of flower bud in each bracteole (Jong, 1995). Based on the research done by Jong (1995), the flower has its own daily opening peak hour that is between 1100 to 1400 hours and the duration for the entire inflorescence to bloom is about 30 days for the staminate flowers and 50 days for hermaphrodite flowers.

**Figure 1: Showing the flower or inflorescence of sago.** Flower initiation act as an indicator for the starch formation in the trunk.

Extensively, overlapping in the opening of staminate and hermaphrodite flowers does occur despite the sago palm flowers are a protandrous type (Saidin, 1993) which means the male flower mature first compared to the female flower. This process will result in cross-pollination (Saidin, 1993). Eventually, the flowers will form into seedless fruits which are round in shape and pale-yellow in colour. Seedless fruits show a significant discovery that "the pollen and pistil of sago palms may be self-incompatible" (Jong, 1995).

## 2.2. *Synthesizing cDNA molecule*

In order to construct cDNA library, it is crucial to have abundant copies of cDNA molecules. It starts off with extracting mRNA from the selected organisms. Extracted RNA in eukaryote cells contain a mixture of molecules like ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA) and snRNA (Russell, 1992) which by far the most abundant amount in a cell are tRNA and mRNA (Malacinski, 2003). However, mRNA strand has a unique feature that can be used to identify and isolate mRNAs from other unwanted RNA molecules. mRNA molecules contain a special poly (A) tail that can be purified using a column to which short chains of deoxythymidylic acid (oligo(dT) chains) have been attached. While the RNA passes through the column, the poly (A) tail of mRNA would make complementary base pairs with the oligo(dT) chains and thus trapping the mRNAs inside the column (Russell, 1992). Later, the captured mRNA is released by increasing the ionic strength of the buffer to disrupt Hydrogen bonds (Russell, 1992) and will be collected in a separate container.

Once the isolation of mRNA population is done, double-stranded cDNA copies are synthesized in vitro, using the enzyme reverse transcriptase (RT) (Russell, 1992). There are two different forms of reverse transcriptase that are available commercially: (1) avian reverse transcriptase; and (2) murine reverse transcriptase (Sambrook *et al.*, 1989). Avian reverse transcriptase is obtained from birds infected with avian myeloblastosis virus (AMV) or a recombinant *E. coli* which expressed the AMV *pol* gene (Brown, 1991). As for the murine reverse transcriptase, it is isolated from recombinant *E. coli* which expresses the reverse transcriptase gene of the Moloney Murine Leukaemia Virus (MMLV) (Sambrook *et al.*, 1989). AMV-RT

requires an incubation temperature at 42°C whereas MMLV-RT enzyme should be incubated at 37°C. Both of the enzymes are widely used in synthesizing first strand cDNA (Brown, 1991).

The synthesis of the first strand cDNA begins with the hybridisation of short oligo(dT) chains to the poly (A) tail at the 3' terminus of each mRNA strand (Russell, 1992). These oligo(dT) chains act as primers for reverse transcriptase to make a complementary DNA copy of the mRNA strand (Russell, 1992). Next is the replacement reaction to synthesize the second DNA strand which involves the use of RNase H, DNA polymerase I and DNA ligase (Russell, 1992). This replacement reaction was first introduced by Okayama and Berg (1982) and later modified by Gubler and Hoffman (1983) (Sambrook *et al.*, 1989). Each of the enzymes has their own function where RNase H is responsible in degrading the RNA strand in the DNA-mRNA hybrid (Russell, 1992). The mRNA strand is partially degraded by RNase H leaving behind a series of short RNA primers (Sambrook *et al.*, 1989) that are used by DNA polymerase I to synthesis new DNA fragments (Russell, 1992). According to Sambrook *et al.* (1989), the reaction has three major qualities: (1) it is very efficient; (2) the products of the first-strand reaction can be used directly without further treatment and purification; and (3) it does not involve S1 nuclease enzyme in order to cleave the single-stranded hairpin loop – "a reaction that is difficult to control and results in a great loss of cDNA" (Sambrook *et al.*, 1989).

## 2.3. *Constructing cDNA library*

cDNA library is a collection of cloned cDNA copies. It is used for comparing gene activities in different cell types of the same organism (Russell, 1992). Since the cDNA is transcribed only from certain parts of the genome, cDNA library is relatively smaller than a complete genomic library (Griffiths *et al.*, 1999).

To begin constructing a cDNA library, the cDNA molecules that have been synthesized must be cloned inside a vector (Russell, 1992). Before that, the cDNA molecules must first be added with linkers (Russell, 1992) or adaptors. But, adaptors are more preferable compared to linkers because adaptors do not require any cleavage with restriction enzyme after they are ligated to the cDNA molecules (Sambrook *et al.*, 1989).

Adaptors are short, double-stranded oligonucleotides that have both blunt and cohesive ends in a single molecule. The blunt end at one side is ligated to the double-stranded cDNA whilst the cohesive end at the other side is supposed to be ligated to a complementary base pair of the vector (Sambrook *et al.*, 1989). The ligation process is done by T4 DNA ligase. T4 DNA ligase is the only ligase enzyme that works efficiently in joining blunt-end terminal under a normal reaction condition (room temperature <30°C). Besides that, T4 DNA ligase can also perform cohesive-end ligation which is normally done at 12-15°C (Ausuble *et al.*, 1999).

Once the cDNA molecules have been added with adaptors, then the cDNA are ready to be cloned inside a vector, using either plasmid or phage vectors. Phage-cDNA libraries are a lot easier to screen at higher densities (Davis *et al.*, 1994) using both nucleic acid and antibody probes (Sambrook *et al.*, 1989) apart from they are rather easy to plate, amplify and store if they are compared to plasmid libraries.

However, at certain circumstances, plasmid vectors are more favourable in the efficiency of generating recombinant clones (Davis *et al.*, 1994).

The usage of phage vector will be emphasizing on lambda phage vectors. There are several types of lambda phage vectors that can be used to clone cDNA such as λZAP, λgt10, λgt11 etc. but only λZAP will be discussed in detail. λZAP vector is a new generation of vectors, which has the properties of insertion from a λ phage and the biology of M13 (Walker & Rapley, 1997) and it is constructed in such a way that could alleviate the scope of potential sites for cloning (Sambrook *et al.*, 1989). This can be achieved with the presence of six unique restriction sites within the polycloning region namely *Xho*I, *Eco*RI, *Spe*I, *Xba*I, *Not*I and *Sac*I sites (Sambrook *et al.*, 1989). According to Walker & Rapley (1997), "λZAP also has many of the advantageous features of λ phage vectors, such as:

i. High cloning efficiency, which thereby increases the chance of recovering the desired fragment from a library;
ii. A multiple cloning site capable of retaining up to 10kb inserts;
iii. Insertional inactivation of β-galactosidase for Xgal blue/white clone selection;
iv. Expression of β-galactosidase fusion proteins analogous to λgt11." (Walker & Rapley, 1997, p.183).

Nevertheless, λZAP also has a few disadvantageous which include:

i. Selection of inserted vector is based on colour selection rather than biological selection which make it quite unreliable for interpreting results (Davis *et al.*, 1994).

ii. Purification of probes has to be done in order to eliminate plasmid, lambda phage and filamentous phage sequence which are included in λZAP vectors (Davis *et al.*, 1994).

Ultimately, the principle of this vector is fairly simple. The recombination process occurs at the cloning site which is located at the *lac*Z gene. When the DNA is inserted into the vector, the *lac*Z gene becomes inactivated making it appears 'clear' when plated onto *E. coli* XL1-Blue plating cells. In contrast, blue plaques will appear onto the XL1-Blue cells when recombination does not occur because there is no inserts in the cloning site which means the *lac*Z gene is not interrupted and remain activated.

*2.4. Calculating number of clones required*

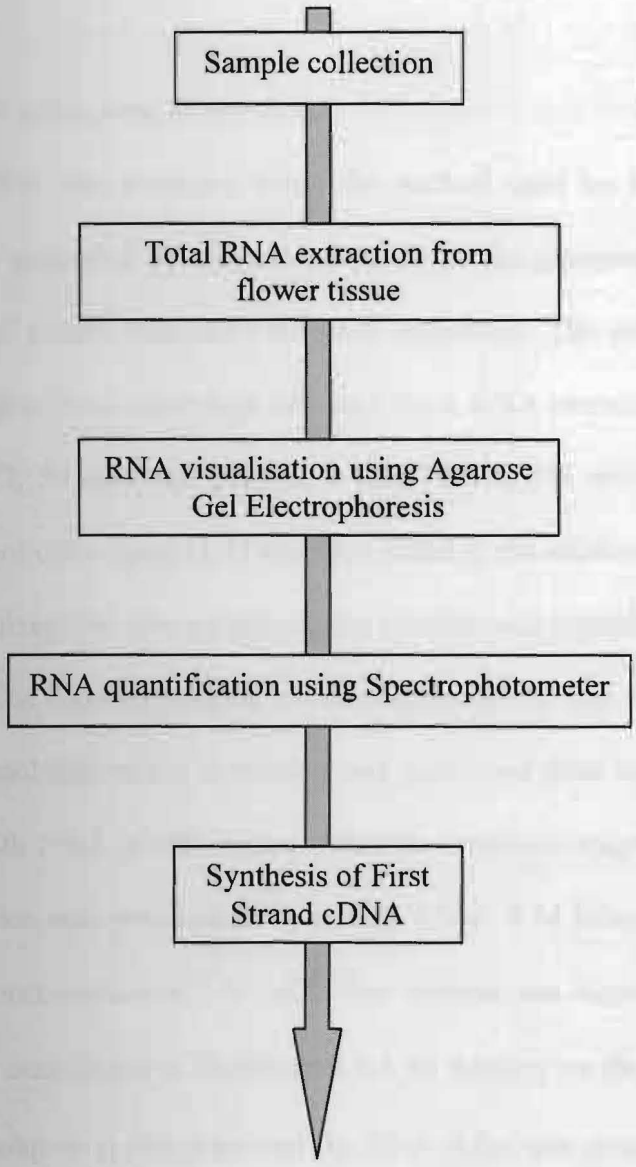
This is necessary to ensure that there is sufficient number of copies in order to construct cDNA library. The formula is as shown below (Sambrook *et al.*, 1989):

$$N = \frac{\ln (1-P)}{\ln (1-1/n)}$$

N = Number of recombinants needed
$P$ = Probability desired
1/n = Fractional proportion of the total

## 3. MATERIALS AND METHODS

The methods used in constructing cDNA library are simplified in a schematic diagram as shown below:



### 3.1. *Sample collection*

Samples (flower tissue) of *Metroxylon sagu* was collected around the areas of Kuching and Kota Samarahan. The samples were rinsed first before preserved in liquid nitrogen. The preserved flower tissue was stored at -80°C in laboratory prior to use.

### 3.2. *Total RNA extraction from flower tissue*

Total RNA was extracted using the method used by Hussain (2003). The samples were grounded by mortar and pestle in the presence of liquid nitrogen. About 2.5g of sample was used for each extraction. The powdered sample was transferred into a 30mL centrifuge tube and 15mL RNA extraction buffer was added (150 mM LiCl, 50 mM Tris pH 9.0, 5 mM EDTA, 5% w/v SDS) and vortexed. 15mL of phenol/chloroform (1:1) was then added to the solution and vortexed again. After it was mixed, the tube containing the solution was centrifuged for 5 minutes at 13,000 rpm. The aqueous solution (containing the RNA) was transferred into a new tube. The phenol/chloroform extraction was performed three times followed by two extractions with 15mL of chloroform. After the extraction stage, RNA present in the aqueous solution was precipitated by adding 3.3mL 8 M lithium chloride (LiCl) to give a final concentration of 2 M LiCl. This mixture was incubated for overnight at 4°C and then centrifuged at 10,000 rpm for 30 minutes on the next day. This time the aqueous solution is discarded and the RNA pellet was resuspended in 400μL of sterile distilled water ($dH_2O$). The RNA was precipitated again in 1.0mL of 100% (v/v) ethanol and 40μL 3M sodium acetate, and was incubated on ice for 20 min. The precipitated RNA was centrifuged 18,000 rpm for 30 minutes and then followed

by washing twice with 70% (v/v) ethanol. Finally, the total RNA pellet was air-dried and resuspended in 100μL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and was stored at -80°C.

### 3.3. *Visualization of RNA molecules*

Agarose gels were prepared by boiling 0.6g agarose in 6ml of 10x TAE buffer and 54mL of distilled water. The melted gel was left to cool under running tap water before pouring it into the tray. The hardened gel was pre-run at 140v for 5 minutes before loading in the samples. 5μL of total RNA was used for each sample. Samples were mixed with 1μL of loading dye. After that the samples were left to run as usual for 2½ hours in 1x TAE buffer.

### 3.4. *RNA quantification using Spectrophotometer*

After visualization, the isolated RNA must be quantified in order to identify the concentration and purity of the sample. The quantification procedure is done using an Ultrospec® 1100 Pro spectrophotometer machine. 10μL of each sample were aspirated into a cuvex and the rest of the cuvex was filled with 490μL of sterile distilled water. Then, the samples were analyzed one at a time by the machine.

### 3.5. *First strand cDNA synthesis*

This protocol is provided by Hwang (2004) with a little bit of modification to suit the condition for the Avian Myeloblastosis Virus (AMV) reverse transcriptase enzyme. Firstly, 2μL of total RNA was placed in a microcentrifuge tube. Then, 2μL of oligo(dT) primer at the concentration of 10μM was added into the same tube

together with 8 μL of DEPC water and mixed well. Next, the tube containing the mixture was incubated for 5 minutes at 70°C and then was placed on ice for 5 minutes. After chilling on ice, 4μL of Promega® 5x Reaction buffer was added together with 2μL of solution containing each dNTP at 10mM. The mixture was incubated again at 42°C for 5 minutes. Subsequently, 3μL of Promega® AMV reverse transcriptase was added into the tube followed by incubation step at 42°C for 60 minutes. After that, the mixture was incubated at 70°C for 10 minutes so as to terminate the synthesizing process. Finally, the tube was kept in -20°C for later use.

# 4. RESULTS AND DISCUSSIONS

## *4.1. Total RNA extraction from flower tissue*

Isolation of RNA is a very vital first step in ensuring that the construction of cDNA libraries to become successful. But, extracting RNA the conventional way, is very time consuming and laborious. Despite the difficulties to be dealt with, the process of extracting total RNA from flower tissue using phenol extraction method adopted from Hussain (2002) had eventually been a great success. Even so, only two large scale extractions yielded sufficient amount of total RNA as requested. Previously, small scales RNA extractions were carried out but fail to retain any RNAs when analyzed using Agarose Gel Electrophoresis. Limited amount of fresh samples may be the factor of failing to sustain intact RNAs. Calculation to determine the amount of extractable RNA for the first and second extraction was therefore:

| Extractable RNA = Concentration x Total volume |
|---|

First extraction:

Extractable RNA = 4.207 μg/μL x 120μL

= **504.84μg**

Second extraction:

Extractable RNA = 2.303 μg/μL x 490μL

= **1128.47μg**

Phenol extraction method is widely applicable and useful in extracting total RNA for the reason that most of the RNA molecules in cells co-exist with proteins (to form complexes called ribonucleoprotein or RNP) (Slater, 1983). Phenol acts as a protein denaturant to degrade protein from RNP complex (Slater, 1983) and the protein is extracted in the phenol layer. Since phenol and water does not mix together, the phenol layer is discarded leaving the RNA in the aqueous layer (Darbre, 1988). The phenol method is then followed by two extractions with chloroform alone which is responsible in removing any traceable amount of phenol (Darbre, 1988).

Nevertheless, in the final step of extraction, there was some obscurity in dissolving the pellet thoroughly in TE buffer for storage purposes. This may be due to inadequate drying of the pellet from ethanol residue or may be overdried. But to McCaughern-Carucci's (n.d) opinion, RNA should be resuspended in formamide rather than in water or ethanol. RNA pellet could dissolve instantly in 100% formamide and the RNA is protected from RNases even is stored at 4°C (McCaughern-Carruci, n.d).

4.2. *RNA quantification*

When the total RNA undergone for quantification, the result indicates that the presence of impurities was quite high. The results obtained were shown in the tables (Table 1 and Table 2) below:

| First Extraction | |
|---|---|
| **Absorbance** | **RNA concentration** |
| 230 nm : 10.00 A<br>260 nm : 2.220 A<br>280 nm : 1.887 A<br>320 nm : 0.117 A | **Conc.** : 4.207 µg/µl<br><br>260/280 nm : 1.188<br>260/230 nm : 0.213 |

**Table 1: Shows the results obtained from the first extraction.**

| Second Extraction | |
|---|---|
| **Absorbance** | **RNA concentration** |
| 230 nm : 10.00 A<br>260 nm : 1.717 A<br>280 nm : 2.260 A<br>320 nm : 0.565 A | **Conc.** : 2.303 µg/µl<br><br>260/280 nm : 0.680<br>260/230 nm : 0.122 |

**Table 2: Shows the results obtained from the second extraction.**

Normally, the readings should range between 1.8 and 2.0 because ratios lower than 1.8 signify that the sample was contaminated with phenol or protein (Brown, 2002, cited in Sundaraj, 2004). The data obtained for absorbance at 260/280nm was very low, only falls around the range of 1.188 to 0.680. According to Neill (1993) it always had been difficult to obtain high yield of RNA from plants. This is because RNA extraction can be easily contaminated with contaminants such as nucleases, starch and phenolic compounds (Neill, 1993).

Loomis (1974) stated that phenolic compounds were likely to form covalently linked quinones when oxidized and strongly bind to nucleic acids (cited in Salzman *et al.*, 1999). When binds to nucleic acids, it would result in the formation of viscous, insoluble material which intervenes with the isolation stage (Murillo *et al.*, 1995) as shown in Figure 2. In addition, the inability to break down every cell

because of the presence of cell wall was also a factor in reducing the yield of RNA (Neill, 1993).

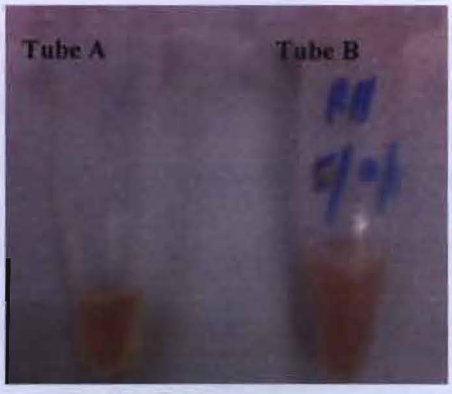



**Figure 2: Insoluble pellet at final step of extraction.** Tube A shows a clearer pellet solution. Tube B contains insoluble, viscous material which intervenes with Spectrophotometer reading.

In the end, lack of expertise and poor handling might also contribute in achieving low yield of total RNA. More to the point of preserving high yield total RNA, sharing equipments and glasswares with other affiliates that do not work with RNA could also add to RNase contamination and thus increases RNA degradation.

So as to avoid from any contaminations, precaution steps should be practiced at all times. All solutions, glassware, and plasticware should be specially treated accordingly (Kingston, 1992). Gloves should be worn and changed frequently throughout all RNA preparation because "hands are a major source of contaminating RNase" (Kingston, 1992). A small amount of RNase present in a RNA preparation would give a hard time in retaining RNA molecules. Besides that, all glasswares should be baked at 300°C for 4 hours because autoclaving itself will not fully inactivate many RNases (Kingston, 1992).

*4.3. RNA visualization*

RNA that has been extracted was analyzed by Agarose Gel Electrophoresis (1% agarose in TAE buffer) as shown in Figure 3. RNA molecules migrated to the positive electrode during electrophoresis because of their polyanion properties (Slater, 1983). The migration of RNA is highly dependent on the size of RNA where smaller molecules move faster than bigger molecules. The RNA samples were run on gel for approximately 1½ hours or when the loading dye has reached the middle section of the gel. This is to prevent the samples from overrunning the gel.

RNA visualization is crucial to determine the existence and the quality of the RNA. The RNA was not of good quality, as judged by the slight appearance of the two ribosomal RNA bands (25S and 18S) indicating RNA degradation occurred during extraction. The RNA prepared was also contaminated with proteins and this can be obviously seen from the smearing along the lanes. Nevertheless, Darbre (1988) pointed out that smearing down the gel track was caused by serious broken down of RNAs.

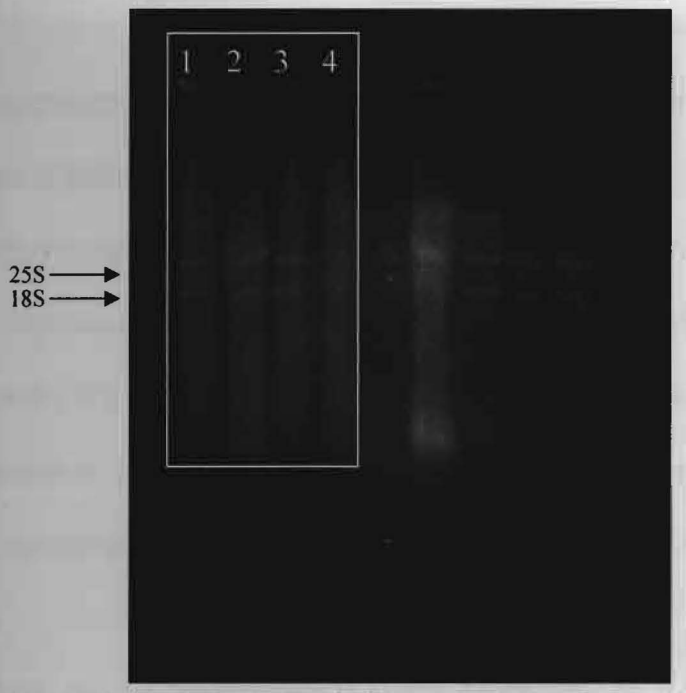



**Figure 3: Analysis of RNA extracted from flower tissue.** Agarose gel analysis of total RNA prepared from flower tissue was shown in Lane 1, 2, 3, and 4. The gel was stained with EtBr and photographed under UV light.

On the other hand, extracted RNA that was analyzed by formaldehyde-agarose gel does not yield any results. This may because of the small amount of RNA extracted and the dissolved RNA was too diluted. According to a protocol written by Perbal (1988), the dried pellets must be resuspended in distilled water to give a final concentration of 10μg RNA in 9.3μL.

### *4.4. First strand cDNA synthesis*

The first strand cDNA synthesis was carried out using total RNA instead of pure mRNAs. Lacking of time and unobtainable oligo(dT) columns prohibits the purification process of mRNA from total RNA. In spite of that, the oligo(dT) which act as primer would recognize and compliment with the poly(A) tail at the 3' end of most mRNAs and the process of synthesizing first strand cDNA can be continued. Promega® AMV reverse transcriptase enzyme was used to generate the first strand